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Kinetics of the toxic action of Pb^{2+} on *Rhizopus nigricans* as studied by microcalorimetry[1]

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Abstract

The microcalorimetric bioassay for acute cellular toxicity is based on metabolic heat production from cultured cells. Microcalorimetry is a quantitative, inexpensive, and versatile method for measuring acute toxicity. The biological response to toxicants is the inhibition of the heat production rate in cells and toxicity is expressed as the concentration of toxicant that is 50% effective in this inhibition (IC_{50}). In this paper, the effect of Pb^{2+} on *Rhizopus nigricans* growth was investigated at 25°C. The relationship between growth rate constants (k) and concentration of Pb^{2+} (C) is $k = 0.04226 \exp[-3.636 \times 10^{-5} (C + 177.0)^2]$, and IC_{50} is 47.5 μg ml^{-1}. These signals are readily obtained by an LKB 2277-204 heat conduction microcalorimeter. This system can therefore measure acute toxicity for a broad range of toxicants having target sites in various cellular metabolic pathways.

Keywords: Microcalorimetry; Toxicity measurement; Pb^{2+}; *Rhizopus nigricans*; Thermokinetics

1. Introduction

In recent years, growing concern has been expressed about chemicals such as heavy metals and organic compounds because of their possible effects on the environment and threats to human health [1]. However, agriculture, industry, and medicine rely on the use of these chemicals. A practical resolution of these conflicting interests requires accurate toxicological information. Acute toxicity tests are the first step in determining such information. An acute toxicity study can establish the relationship between the dose of a toxicant and the effect of it on the tested organism. Results can be presented as plots of toxicant concentration versus effect, for example, mortality rate, growth rate, inhibitory rate, etc. Toxicities of substances are expressed as LC_{50}, IC_{50} or EC_{50} values. The accurate measurement of the effects of potentially toxic materials such as heavy metals and complex industrial effluents on aquatic systems depends on the reproducibility of acute toxicity tests [1].

Bioenergetic investigations which should be most important in the field of the assessment of harmful

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properties of substances in ecotoxicology [2] are closely related to the applicability of the direct calorimetry in biology because there is scarcely another method to analyze metabolic activities possessing such general validity as calorimetry [3].

Calorimetry has also been particularly useful in monitoring cellular metabolism, and heat measurements have long been used to study metabolism in cells and whole organism [1,4]. Microcalorimetry has been useful in measuring the effects of various substances and culture conditions on metabolism. Miles and Beezer demonstrated that microcalorimetric studies of bacterial growth reveal temporal details not observable by other techniques [5]. Microcalorimetry can also be used to study the metabolism of mitochondria and the effects of toxicants on mitochondrial metabolism [6–10]. Thermogenic curves contain a lot of kinetic information. By analyses of the thermogenic curves, we have studied the effect of toxic agents on microbes [11] and mitochondrial metabolism [12], and obtained considerable kinetic data. Binford determined microcalorimetrically the antibiotic sensitivities of 15 different strains of clinically important bacteria [13]. Tan et al. studied the effect of quartz particles on alveolar macrophages from guinea-pigs, and demonstrated that the microcalormetric method could be used as a short-term cytotoxic test for measuring potentially toxic agents in our environment [14].

This paper describes briefly the application of the microcalorimetric method to environmental sciences. It is a quantitative, inexpensive and versatile method for measuring acute toxicity. Heat production in a cell suspension is measured by the thermopile of a LKB 2277 heat conduction stopped-flow microcalorimeter. The inhibition of biochemical reactions in the cells by some toxicant results in a decrease in growth rate constants and heat production.

2. Experimental

2.1. Materials

2.1.1. Cells and reagents

Rhizopus nigricans (CCTCC AF91113), was provided by the Chinese Center for Type Culture Collections, Wuhan University, Wuhan 430072, P.R. China.

Analytical reagent grade $Pb(NO_3)_2$ was supplied by Shanghai Second Reagent Factory, Shanghai, P.R. China.

2.2. Cultural medium

R. nigricans was grown on a potato medium. This was made by taking 200 g potato (without peel) cut into small pieces, then cooked for about 40 min and filtered. The sediment was discarded. 20 g glucose was then dissolved in the clear solution, which was then made to 1000 ml with distilled water and sterilized at 120°C for 20 min.

2.3. Calorimeter

The LKB 2277 Bioactivity Monitor which is a type of heat-flow microcalorimeter, was used to determine the metabolism of cells. It is designed to monitor continuously a wide variety of processes and complex systems over the temperature range 20–80°C. A schematic representation of the calorimetric system is shown in Fig. 1. Each measuring cylinder normally contains a sample and a reference in separate measuring cups (twin system). The heat output from the sample flows from the thermoelectric detector to the large heat sink (in close contact with the water bath). In response, the detector produces a voltage which is proportional to the power output from the sample. In order to minimize the systematic error and disturbance effect, a differential or twin detector system is used. This system is very sensitive, the detection limit being 0.15 μW and the baseline stability (over a period of 24 h) being 0.2 μW. There are three operating modes for the LKB 2277 Bioactivity Monitor: ampoule mode, flow-through mode and flow-mix mode.

In the monitoring system, two precision resistors for electrical calibration are built into each measuring cylinder and one for each detector. When a known current is passed through the appropriate resistor, the detector can be calibrated easily. Other methods for calibration are suitable internally calibrated radioactive sources and chemical reactions. Using one of these techniques, a calorimetric constant can be determined. The time constant (τ) of this instrument is about 120 s. The performance of this instrument and the details of is its construction have been previously described previously [15].

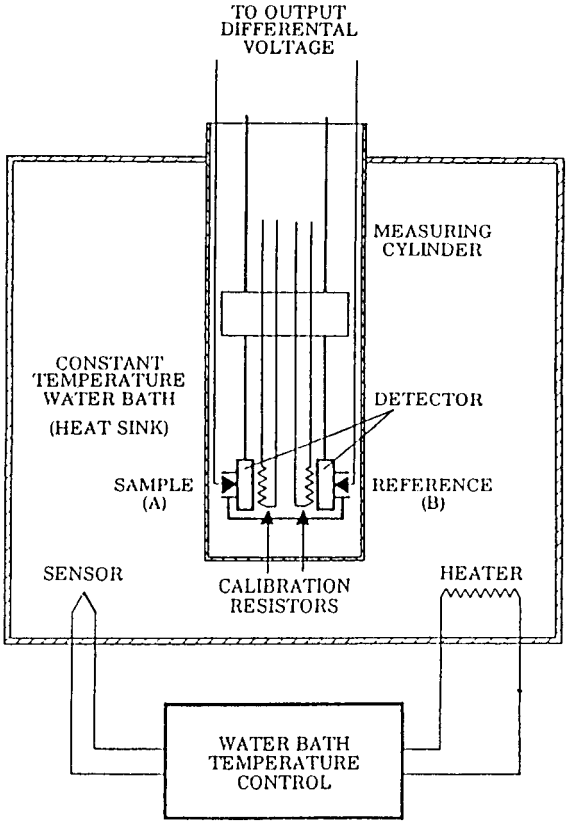

Fig. 1. Simplified operation diagram.

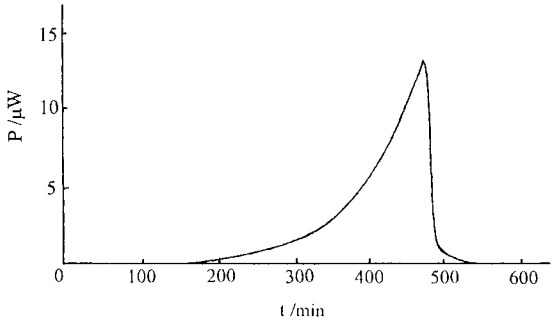

Fig. 2. Thermogenic curve of *R. nigricans* growth at 25°C.

2.4. Preparation of the sample

In this type of experiment, the solution of $Pb(NO_3)_2$ was prepared in sterilized distilled water and were prepared freshly every time. In the beginning of the experiment, *R. nigricans* was inoculated in the prepared potato culture medium, initially containing 1×10^6 cells ml^{-1}, and the cells used were suspended in the potato culture medium, then the fresh $Pb(NO_3)_2$ solution was added into the cell suspension.

2.5. Experimental procedure

The flow-cell was cleaned and sterilized as follows: (1) sterilized distilled water was pumped through the system for 30 min at a flow rate of 40 ml h^{-1}; (2) a 0.1 mol l^{-1} solution of HCl was pumped through the system for 30 min at a flow rate of 40 ml h^{-1}; (3) a 75% alcohol solution was pumped through the system for 30 min at a flow rate of 25 ml h^{-1}; (4) a solution of 0.1 mol l^{-1} NaOH was pumped through the system for 30 min at a flow rate of 40 ml h^{-1}; (5) sterilized distilled water was again pumped through the system for 30 min at a flow rate of 40 ml h^{-1}.

Once the system was cleaned and sterilized, sterilized distilled water was pumped through the system at a flow rate of 10 ml h^{-1} to run the baseline. After a stable baseline had been obtained, the cell suspension, containing *R. nigricans* and Pb^{2+}, was pumped into the flow-cell (0.6 ml) by the aid of an LKB 2132 perplex peristaltic pump at a flow rate of 50 ml h^{-1}. When the flow-cell was full, the pump was stopped, and the monitor recorded the thermogenic curves of the growth of *R. nigricans* continuously at 25°C.

When the pen of the chart recorder had returned to the baseline and stabilized, *R. nigricans* growth had ended. The thermogenic curve of *R. nigricans* growth at 25°C are shown in Fig. 2. If necessary, further calibration was done after a stable baseline has been obtained.

3. Results

3.1. Calculation of the growth rate constant of Rhizopus nigricans

In the log phase of growth, the cell is growth exponentially [11,16]. If the cell number is n_0 at time 0, and n_t at time t, then

$$n_t = n_0 \exp(kt) \quad (1)$$

k is the growth rate constant. If the power output of

Table 1
Rate constants (k) for the growth of *Rhizopus nigricans* at 25°C

	Experiment number					
	1	2	3	4	5	6
k (min^{-1})	0.01395	0.01340	0.01349	0.01500	0.01321	0.01207
R	0.99713	0.99786	0.99656	0.99721	0.99558	0.99868

each cell is w, then

$$n_t w = n_0 w \exp(kt) \quad (2)$$

$$P_0 = n_0 w \quad \text{and} \quad P_t = n_t w$$

giving

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \quad (3)$$

The thermogenic curves of the log phase of growth correspond to Eq. (3). So, making use of the data $\ln P_t$ and t taken from the curves to fit a linear equation, one can obtain the growth rate constant (k). The rate constants (k) of *R. nigricans* growth are shown in Tables 1 and 2.

From the data in Table 1, it is apparent that $k = 0.01352 \pm 0.00087\ min^{-1}$. All of the correlation coefficients are larger than 0.9950, indicating a good reproducibility and correlationship.

3.2. Relationship between t_P and the concentration of Pb^{2+}

All power–time curves change with an increase in the mass of Pb^{2+}, resulting in a shift to longer times, and becoming very broad. For this system, a quantitative analysis can be fitted to a mathematical model. Peak times of growth thermogenic curves (t_P) are correlated to the initial concentration of Pb^{2+}. Using the values of t_P, the relationship between t_P and concentration of Pb^{2+} is

$$t_P = 474.6 + 4.53C, \quad \text{and} \quad R = 0.9936 \quad (4)$$

By a linear regression, a straight line was obtained from the values of t_P versus concentration plot. This linearization showed a best fit for the data in Table 2.

3.3. Relationship between P_m and growth rate constants (k)

The addition of $Pb(NO_3)_2$ caused a decrease of the maximum heat production rate (P_m) and growth rate constants (k). Values P_m are correlated to the growth rate constants (k). Using the values in Table 2,

$$P_m = 0.38 + 925.3k, \quad \text{and} \quad R = 0.9960 \quad (5)$$

3.4. Relationship between k and concentration of Pb^{2+}

Analyses of the values of growth rate constants (k) and the corresponding concentration values (C), shows a logarithmic normal distribution, as described in [11,17]. Thus,

$$k = B_1 \exp[-B_2(C + B_3)^2] \quad (6)$$

This model could be adjusted conveniently to this inhibitor, where k is the growth rate constant, B_1, B_2 and B_3 are constants, C is the concentration of Pb^{2+}. Eq. (6) can be rewritten as

$$\ln k = \ln B_1 - B_2(C + B_3)^2 \quad (7)$$

From the least-square method, the values of B_1, B_2 and

Table 2
Rate constants (k) for the growth of *Rhizopus nigricans* with different concentration of Pb^{2+}

Parameters	Concentration (C) of Pb^{2+} (g ml^{-1})					
	0	5	10	20	40	80
k (min^{-1})	0.01352	0.01294	0.01175	0.01002	0.00775	0.00382
P_m (μW)	13.1	12.1	11.1	10.1	7.2	4.0
t_P (min)	470	480	540	580	640	840
I (%)	–	4.3	13.1	25.9	42.7	71.7

B_3 are 0.04226 min^{-1}, 3.636×10^{-5} and 177.0 μg ml^{-1}, respectively. The correlation coefficient, R, is 0.99932, which is very high. We can obtain the relationship between k and concentration of Pb^{2+} as

$$k = 0.04226 \exp[-3.636 \times 10^{-5}(C + 177.0)^2] \quad (8)$$

3.5. Inhibitory ratios and half inhibitory concentrations

High concentrations of Pb^{2+} will inhibit *R. nigricans* growth and the growth rate constant will decrease. So, the inhibitory ratio (I) can be defined as

$$I = \left[\frac{k_0 - k_C}{k_0}\right] \times 100\% \quad (9)$$

where k_0 is the rate constant of the control, and k_C the rate constant for *R. nigricans* growth inhibited by an inhibitor with a concentration of C. When the inhibitory ratio (I) is 50%, the corresponding half-inhibitory concentration of the inhibitor can be represented as IC_{50} . IC_{50} can be regarded as the inhibiting concentration causing a 50% decrease of the *R. nigricans* growth rate constant. Data for I are shown in Table 2. From the data in Table 2, we can see directly that IC_{50} is about 48 μg ml^{-1}. We can also calculate IC_{50} using Eq. (8), in which $k = k_0/2$, and the calculated IC_{50} is 47.5 g ml^{-1}. The values of IC_{50} obtained by these two methods are the same, showing that the action model (Eq. (8)) is correct. This is very significant in toxicology research and should be studied further.

4. Discussion

Direct microcalorimetric monitoring is suitable for ecotoxicological tests with a high degree of reproducibility. This also provides kinetic data. Calorimetry can enhance the accuracy of the determination of the physiological activity of the cultures. The calorimetric determination of toxicity results in lower as well as higher values of standard data within the range of variances of toxicity determinations by different laboratories using one standard method [3].

Direct microcalorimetry of continuous cultures is a fast response technique to determine the toxic properties of chemicals and seems to be qualified for a feed forward control strategy within biological sawage treatment [3]. Thus, microcalorimetry could be helpful in the safeguarding of our environment by improving the performance and the operational safety of wastewater treatment plants.

In this toxicant (Pb^{2+}) study, the percent inhibition of *R. nigricans* growth increased with Pb^{2+} concentration. Factors that determine the characteristics of a dose-response curve are the toxicant's mode of action in cells, its number of target sites, and its affinity for those target sites. All the cellular level, metals can bind sulfhydryl groups on membrane proteins, resulting in cross-linking and inactivation. This changes cell membrane permeability and disrupts transport of nutrients and waste across the membrane. The toxicity of a metal for cells depends on its oxidation state, speciation, and the stability and solubility of its compounds. Some studies showed a correlation between toxicity and sulfhydryl affinity, suggesting that the cross-linking of membrane proteins is a major factor in the toxic effects of materials [18].

Calorimetry has proven a useful tool for measuring the energy flow in natural samples. It is adaptable to toxicity studies in any cell type. The advantage of calorimetry is that it measures the total thermal energy flow. Under certain conditions the latter can also be determined indirectly using respirometry. In contrast to calorimetry, however, respirometry is restricted to aerobic conditions and metabolism in which oxygen is the final electron acceptor. The advantage of calorimetry in this respect is its non-specificity. By combining calorimetry and other specific methods, several different and important goals may be reached in studying the energy flow in natural environments [19].

New methods and approaches are needed in toxicity studies and for the development of toxicity test systems. Several of the presentations show or indicate the potential of the applicability of calorimetry combined with other methods to determine the influence of toxicants or of eutrophication on different ecosystems. We believe that this is one of the most important and powerful applications of our present and future efforts.

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